Increased Resistance *in Vitro* of *Pseudomonas Aeruginosa* to Chlorhexidine

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(Received June 19, 1979)

ABSTRACT

The tolerance of four strains of *Pseudomonas aeruginosa* to chlorhexidine increased 8 to 32 times during 21 passages every other day in nutrient broth containing subinhibitory concentrations of the disinfectant. The cells from the 30th subcultures died more slowly in either 0.5 or 0.02% solution of chlorhexidine than those of the parent strains. Likewise, the minimal inhibitory concentrations were greater with acrinol for the bacteria from the 30th subcultures than for those of the parent strains. On the other hand, no significant differences were observed between the parent strains and those from the 30th subcultures in minimal inhibitory concentrations of ampicillin, carbenicillin, dibekacin, amikacin, polymixin B and benzalkonium chloride. The bacteria that had acquired resistance after 30 subcultures in the presence of chlorhexidine were further subcultured for another 30 passages in a disinfectant-free nutrient broth. In three of the four strains tested, the acquired resistance to chlorhexidine was reduced to one-fourth to one-eighth, but it was unchanged in one strain.

Key Words: pseudomonas aeruginosa; chlorhexidine; stable mutant

INTRODUCTION

The appearance of resistance in bacteria to various chemotherapeutic agents has become an important clinical problem. In addition, it has been reported that some bacteria develop resistance to antiseptics such as silver nitrate[1], phenol[2], proflavine[3], quarternary ammonium compounds[4,5], and chlorhexidine (CHX)[6]. Such bacteria may cause nosocomial infections.

CHX has a marked *in vitro* antibacterial activity against a wide range of micro-organisms[7] with little irritation to tissues. It is widely used in hospitals as a disinfectant. Therefore, the development of bacterial resistance to CHX may lead to nosocomial infections. In fact,

infections resulting from the use of CHX preparations contaminated with pseudomonads have been reported[8-11].

Pseudomonas aeruginosa is the main causative organism of nosocomial infection[12]. It seems, therefore, of great importance to clarify the processes of acquirement and loss of resistance to CHX by *P. aeruginosa*. The present paper describes the difference in the time required to kill the parent strains and the evolved resistant cells in aqueous CHX solutions, and the differences in sensitivity to several drugs of the parent strains and their resistant cells. The information will be useful for prophylaxis of nosocomial infections.

MATERIALS

Bacterial strains: Four strains of *P. aeruginosa* (IFO 3919, 3923, 12582 and 12689) were supplied by the Institute for Fermentation, Osaka, Japan, and are refferred to herein as strains Ⅰ, Ⅱ, Ⅲ and Ⅳ, respectively.

Media: Nutrient broth and agar were the products of Eiken Chemical Co. Nutrient agar (Difco) was used for determination of minimal inhibitory concentrations (MIC) of fosfomycin.

Chemicals: Antiseptics and antibiotics used were CHX digluconate (Hibitane®, Sumitomo Chemical Co.); ampicillin, fosfomycin, dibekacin (Meiji Seika Co.); carbenicillin, polymixin B (Pfizer Taito Co.); amikacin (Banyu Pharmaceutical Co.); acrinol (Nakarai Chemical Co.); and benzalkonium chloride (Hoei Yakko Co.).

RESULTS

Acquirement of resistance:

The development of resistance to CHX was examined by successive transfer (every other day) of bacteria to nutrient broth containing CHX. Five ml of nutrient broth solution of CHX (400 μg/ml) were diluted serially with 5 ml of the broth. To each of the broth, an aliquot (0.1 ml) of 18-h broth culture was inoculated. Each tube was incubated at 37°C, and bacterial growth was determined on the basis of turbidity after 48 h. An aliquot (0.1 ml) of the culture containing the maximum concentration of CHX that permitted bacterial growth was then inoculated further into the above series of broth containing varying concentrations of CHX. These procedures were repeated 30 times. The cells from the 30th subcultures of strain I, II, III and IV will be refferred to herein as the cells I^{30th}, II^{30th}, III^{30th} and IV^{30th}, respectively. The minimal inhibitory concentrations (MIC) for strain I and II increased

from 12.5 μg/ml to 400 μg/ml after 21 and 18 passages, respectively. The MIC for strain III increased from 12.5 μg/ml to 200 μg/ml after 21 passages, while that for strain IV increased from 12.5 μg/ml to 100 μg/ml after 15 passages. The results are shown in Table I.

Table I Acquirement of resistance to chlorhexidine by *Pseudomonas aeruginosa*

No. of passages / Strain	Minimal inhibitory concentration (μg/ml)										
	0	1	2	3	4	5	6	7	8	9	10
I	12.5	12.5	12.5	25	25	25	25	25	50	50	50
II	12.5	12.5	12.5	25	25	25	50	50	50	100	100
III	12.5	12.5	12.5	12.5	12.5	12.5	25	50	50	50	50
IV	12.5	12.5	12.5	12.5	12.5	25	25	25	25	25	25

No. of passages / Strain	Minimal inhibitory concentration (μg/ml)										
	11	12	13	14	15	16	17	18	19	20	21–30
I	50	50	50	50	50	100	100	100	200	200	400
II	200	200	200	200	200	200	200	400	400	400	400
III	50	50	50	50	100	100	100	100	100	100	200
IV	50	50	50	50	100	100	100	100	100	100	100

Survival of P. aeruginosa in aqueous CHX solution:

The germicidal effectiveness of CHX against cells before and after acquiring resistance was compared. The cells of 48-h broth culture of each strain, which is free from CHX, were washed three times with sterile saline (0.16 M) by centrifugation at 1400×*g* for 10 min and suspended at approximately 10^8 viable cells per ml. An aliquot (0.1 ml) of each cell suspension was pipetted into tubes containing 5 ml of 0.02 or 0.5% aqueous solution of CHX and incubated for varying periods at room temperature. The concentrations used are those applied to disinfect mucous membrane (0.02%) or skin (0.5%). One loopful samples were taken from the above mixture after 15 and 30 s, and 1, 2, 5, 10 and 30 min and then inoculated into 5 ml of nutrient broth. Following incubation at 37°C for 48 h, bacterial growth was examined through turbidimetry (Table II). The cells of strains I and II did not grow after exposure to the 0.02% CHX solution for 30 min, but the cells I^{30th} and II^{30th} did grow after these treatments. Similarly, the periods required for the suppression of growth after exposure to 0.5% CHX solution were longer in the cells I^{30th} and II^{30th} than those of strains I and II.

Sensitivity to other antimicrobial drugs:

The MIC of 6 antibiotics and 3 antiseptics against the cells of the

Table II Growth of *Pseudomonas aeruginosa* after exposure to chlorhexidine

i) 0.02%aqueous chlorhexidine

Strain \ Time	15 s	30 s	1 min	2 min	5 min	10min	30min
I	+	+	+	+	+	+	−
I^{30th}	+	+	+	+	+	+	+
II	+	+	+	+	+	+	−
II^{30th}	+	+	+	+	+	+	+

ii) 0.5%aqueous chlorhexidine

Strain \ Time	15 s	30 s	1 min	2 min	5 min
I	+	−	−	−	−
I^{30th}	+	+	+	−	−
II	−	−	−	−	−
II^{30th}	+	+	−	−	−

+ : growth
− : no growth

parent and evolved strains were determined by means of a standard dilution method using nutrient agar. The antibiotics used were ampicillin, carbenicillin, fosfomycin, dibekacin, amikacin, and polymixin B, and antiseptics used were acrinol, benzalkonium chloride, and CHX. The cells from 10^{-2} dilutions of 18-h broth cultures of each strain were inoculated on plates of nutrient agar containing various concentrations of antibacterial agents. The MIC were determined after incubation at 37°C for 18 h. The results are shown in Table III. The MIC of CHX obtained by this method agreed with those obtained by the broth method. No significant differences between the parent strains and the cells from the 30th subcultures were observed in the MIC of ampicillin, carbenicillin, dibekacin, amikacin, polymixin B and benzalkonium chloride However, in addition to the increase in resistance to CHX, 4- to 8-fold increase in resistance to acrinol was observed.

Stability of acquired resistance:

In order to study the stability of acquired resistance, the cells from 30th subcultures of each strain were further inoculated successively at 37°C in CHX-free nutrient broth. The results are shown in Table IV. Following the additional 30 passages, the MIC for strain I^{30th} were reduced from 400 μg/ml to 100 μg/ml, that for strain II^{30th} from 400

Table III Sensitivity of *Pseudomonas aeruginosa* to several drugs

Strain \ Drug	Minimal inhibitory concentration (μg/ml)								
	CHX	AB-PC	CB-PC	FOM	DKB	AMK	PM-B	acrinol	benz. ch.
I	6	400	12	25	1.5	3	1.5	200	200
I^{30th}	400	400	6	25	1.5	3	1.5	1600	200
II	12	400	25	12	1.5	3	0.8	200	400
II^{30th}	400	400	12	400	1.5	3	3	800	400
III	12	100	0.4	12	0.8	3	3	200	200
III^{30th}	400	100	0.2	100	0.4	3	3	800	800
IV	12	400	50	100	3	6	1.5	200	400
IV^{30th}	100	100	50	12	3	6	3	800	100

AB-PC: ampicillin, CB-PC: carbenicillin, FOM:fosfomycin, DKB: dibekacin, AMK: amikacin, PM-B: polymixin B, benz. ch.: benzalkonium chloride

Table IV Loss of resistance to chlorhexidine by *Pseudomonas aeruginosa*

Strain \ No. of passages	Minimal inhibitory concentration (μg/ml)										
	0	1	2	3	4	5	6	7	8	9	10
I^{30th}	400	400	400	200	200	200	200	200	200	200	200
II^{30th}	400	400	400	400	400	200	200	200	100	100	100
III^{30th}	200	200	200	200	200	200	200	200	200	200	100
IV^{30th}	100	100	100	100	100	100	100	100	100	100	100

Strain \ No. of passages	Minimal inhibitory concentration (μg/ml)									
	11	12	13	14	15	16	17	18	19	20–30
I^{30th}	200	200	200	200	100	100	100	100	100	100
II^{30th}	100	100	100	100	100	50	50	50	50	50
III^{30th}	100	100	100	100	100	100	100	100	100	50
VI^{30th}	100	100	100	100	100	100	100	100	100	100

μg/ml to 50 μg/ml and that for strain III^{30th} from 200 μg/ml to 50 μg/ml, while there was no change in the MIC for strain IV^{30th}.

DISCUSSION

The MIC of CHX against gram-negative bacteria have been reported to be in the range from 10 to 50 μg/ml[1]. Gillespie et al[13]. reported that the MIC of CHX were above 250 μg/ml against 6 of 13 strains of *Proteus mirabilis* obtained from urine infected during the use of plastic reservoir drainage bags. O'Flynn et al[14]. reported that in 24 of 45 stra-

ins of *P. mirabilis* which apparently survived disinfection by CHX, the MIC of CHX were over 200 μg/ml. The two reports suggest that repeated use of CHX may allow *P. mirabilis* to survive by adapting to grow in 200 μg/ml of CHX, which is the usual concentration applied to disinfect mucous membrane.

In the study of the process of acquirement of resistance by *P. aeruginosa* to CHX *in vitro*, we found the bacteria with MIC of CHX as high as 400 μg/ml in some strains. Since CHX is frequently used in hospitals and *P. aeruginosa* requires little nutrition to grow, acquirement of resistance reported here may also occur in situations where the drug is used.

The resistance to acrinol also increased in the bacteria from 30th subcultures in the medium containing CHX (Table III). This may indicate that these two antiseptics have similar mechanisms of action against *P. aeruginosa*. Although CHX is comparable to benzalkonium chloride in effective concentrations and antibacterial spectrum, the bacteria with increased resistance to CHX did not show increased resistance to benzalkonium chloride.

Four strains of 30th subcultures were subjected to further 30 passages in CHX-free broth, and the acquired resistance to CHX was reduced to one-fourth to one-eighth in three of the four strains tested, but there was no change in the fourth strain. The latter case is probably due to the appearance of a stable mutant resistant to CHX.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Teruko Nakazawa, assistant professor at Yamaguchi University, for her helpful advice.

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